FERMENTATION, ISOLATION, CHARACTERIZATION AND STRUCTURE OF ANTIBIOTIC U-58,431

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A new antibiotic U-58,431 has been isolated from the fermentation broth of *Streptomyces helicus* DIETZ and LI, sp. n. (UC-5837) and the structure of this antibiotic, namely, 6-amino-3,4,5,8-tetrahydro-4,9-dihydroxy-3-methyl-5,8-dioxo-1,4-ethano-1H-2-benzopyron-7-carboxamide has been determined by X-ray crystallography. The antibiotic inhibits a variety of Gram-positive and Gram-negative bacteria *in vitro*. However, it is toxic to mice and does not protect experimentally infected animals when administered at the maximum tolerated dose.

In the course of soil screening for antibiotic-producing microorganisms, a new isolate of *Streptomyces helicus* (UC-5837) was obtained and found to produce a new antibiotic U-58,431. In this paper we shall report the fermentation conditions, the isolation procedure, the biological and chemical properties as well as the crystallographic determination of the structure of this antibiotic.

I. Microorganism

The antibiotic-producing microorganism was a new soil isolate, classified by A. DIETZ and G. P. LI as *Streptomyces helicus* DIETZ and LI, sp. n. (UC-5837).

II. Analytical Methods

The fermentation media and the various fractions obtained during the isolation procedure were analyzed by thin-layer chromatography on silica-gel plates (Analtech, Inc.) developed in chloroform-methanol (9: 1, v/v). In this system, antibiotic U-58,431 had an Rf value of 0.4~0.5 and was visualized with a 366 nm UV-lamp or by bioautography against *S. aureus* (UC-76).

The antibiotic production was determined by the paper disc agar diffusion method employing *S. aureus* (UC-76) as the test organism. The *in vitro* and *in vivo* antibacterial activities were determined according to the methods described by LEWIS *et al.*[1]

The X-ray crystallographic structure determination was done according to the procedure described by DUCHAMP.[2]

III. Fermentation

All fermentations were conducted under submerged culture conditions in 500-ml Erlenmeyer flasks containing 100 ml of culture medium. Seed cultures of *S. helicus* (UC-5837) were prepared in a medium composed of (in g/liter): glucose monohydrate, 25.0 and Pharmamedia (Trader's Oil Mill Co.) 25.0; pH adjusted to 7.2 prior to sterilization. The seed flasks were inoculated with a loop of soil stock prepared according to REUSSER[3] and incubated at 28°C for 3 days on a rotary shaker (250 rpm, 6-cm

stroke). The fermentation medium contained (in g/liter): glucose monohydrate, 15.0; dextrin (Wilson Corn Products, Inc.) 25.0; corn gluten meal (Wilson Corn Products, Inc.) 20.0; oatmeal (Fruen Milling Co.) 15.0 and calcium carbonate, 8.0; adjusted to pH 7.2 with NaOH. The flasks were inoculated with 5% of seed culture and incubated at 28°C on a rotary shaker. Peak antibiotic titers were obtained after 5~6 days of incubation.

IV. Isolation Procedure

Preliminary Isolation Step, Method 1

The pH of the fermentation broth was adjusted to 3 with conc. HCl and the mycelium was removed by filtration. Nine hundred grams of ammonium sulfate were dissolved in two liters of the clear broth and the solution was extracted once with 1,200 ml of *n*-butanol. This step extracted all the bioactivity from the water phase. The *n*-butanol extract was evaporated in a rotary flask evaporator yielding 3.8 g of brown, oily residue.

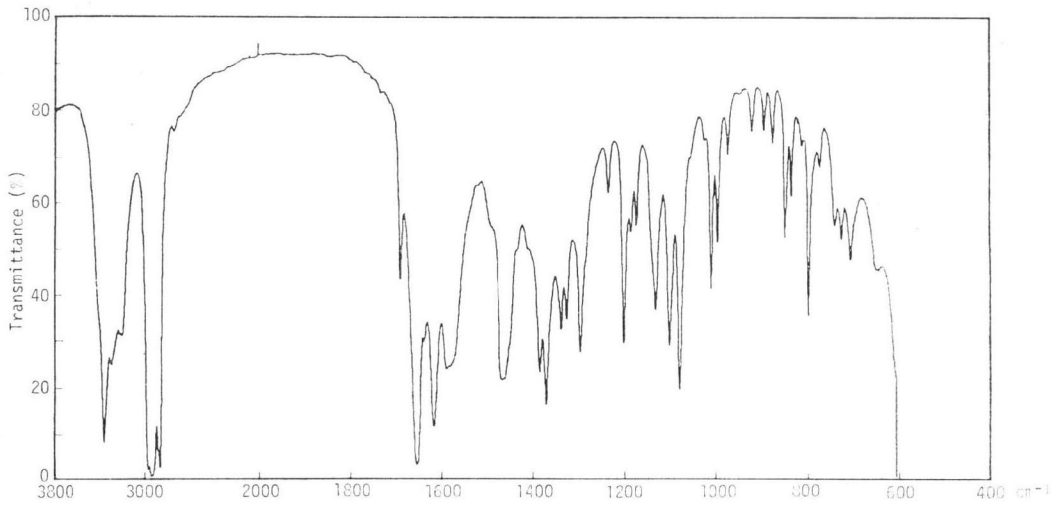
Preliminary Isolation Step, Method 2

Two liters of clear fermentation medium (pH 3) were passed through a column of Amberlite XAD-4 resin (500 ml of resin, 3.5×40 cm). The column was then washed with deionized water until the eluate was colorless. The antibiotic was eluted from the resin with 500 ml of a methanol - water (9: 1, v/v) mixture and the eluate was evaporated to yield 2.6 g of oily residue.

Final Purification Step

The residues obtained by either of the steps above were triturated in boiling chloroform (50 ml per g of residue) and the red chloroform solutions were decanted from the residual oil. The chloroform extract, after addition of 2 g of silica gel, was evaporated at room temperature and the residue was transferred to the top of a silica gel column (200 g; 3.5×35 cm, poured as a slurry in chloroform - methanol, 9: 1). The column was then eluted with the same solvent mixture. Fractions of 100 ml were collected and assayed for bioactivity. The red fractions containing the antibiotic were combined and evaporated to dryness. The residue was dissolved in a minimal amount of boiling chloroform, the solution was

Fig. 1. Infrared spectrum of antibiotic U-58,431 (mineral oil mull).



filtered and the filtrate was refrigerated overnight. The red crystals formed were collected by filtration, washed with chloroform and dried *in vacuo*. The yield was 45 mg of orange-red crystalline antibiotic U-58,431. For analytical purposes, the crude crystals were recrystallized from boiling chloroform.

Anal. Calculated for $C_{13}H_{14}N_2O_6$: C, 53.06; H, 4.80; N, 9.52; M. W. 294.
Found: C, 52.70; H, 5.40; N, 9.45

$M^{+\cdot}$ 294; mp 156~157°C (dec.). Optical activity was displayed, $[\alpha]_D$ +9° (*c* 1, methanol) and UV maxima were observed at 261 nm (ε 13,171) and at 472 nm (ε 1,517). No shifts occurred on addition of acid or base. The infrared spectrum is shown in Fig. 1.

V. Structure Determination by X-Ray Crystallography

Crystal data for antibiotic U-58,431 ($C_{13}H_{14}N_2O_6$) were: monoclinic; space group $P2_1$; $Z=2$;

Table 1. Final atomic parameters, and standard deviations in parentheses.
All values for anisotropic atoms have been multiplied by 10^4. Coordinates of isotropic atoms have been multiplied by 10^3. The form of the anisotropic temperature factors is exp $(-\beta_{11}h^2-\beta_{22}k^2-\beta_{33}l^2-\beta_{12}hk-\beta_{13}hl-\beta_{23}kl)$.

	X	Y	Z	β 11	β 22	β 33	β 12	β 13	β 23
C(1)	9472 (3)	6463 (6)	3083 (2)	47 (4)	102 (9)	22 (2)	−5 (9)	6 (5)	12 (6)
O(2)	10020 (2)	7905 (5)	2338 (2)	58 (3)	90 (5)	34 (2)	−52 (7)	18 (3)	−0 (5)
C(3)	8885 (3)	8224 (6)	1162 (3)	67 (4)	87 (8)	30 (2)	13 (10)	32 (5)	21 (7)
C(3M)	8546 (5)	10207 (7)	1065 (3)	147 (6)	88 (9)	57 (3)	3 (12)	83 (7)	10 (8)
C(4)	7330 (3)	7076 (6)	1123 (2)	47 (4)	85 (8)	19 (2)	24 (9)	15 (4)	15 (6)
O(4)	6247 (2)	7435 (5)	−22 (2)	60 (3)	185 (7)	18 (1)	109 (7)	16 (3)	30 (5)
C(4A)	6713 (3)	7351 (6)	2378 (2)	55 (4)	62 (8)	24 (2)	0 (8)	21 (5)	−3 (6)
C(5)	5058 (3)	7808 (6)	2548 (2)	53 (4)	83 (8)	23 (2)	5 (9)	20 (5)	−7 (6)
O(5)	3942 (2)	8128	1654 (2)	53 (3)	183 (7)	28 (2)	66 (8)	6 (3)	9 (5)
C(6)	4705 (3)	7869 (6)	3923 (2)	67 (4)	60 (7)	26 (2)	−4 (9)	39 (5)	−16 (7)
N(6)	3156 (3)	8200 (6)	3969 (2)	50 (3)	114 (7)	23 (2)	31 (8)	19 (4)	3 (6)
C(7)	5923 (3)	7610 (6)	4974 (2)	55 (4)	62 (8)	25 (2)	−25 (8)	18 (5)	−22 (6)
C(7A)	5519 (3)	7726 (6)	6294 (2)	67 (4)	83 (8)	26 (2)	−19 (9)	25 (5)	−11 (7)
O(7)	4081 (2)	8079 (5)	6455 (2)	73 (3)	160 (6)	28 (2)	37 (8)	41 (4)	−7 (6)
N(7)	6713 (3)	7460 (6)	7285 (2)	72 (4)	175 (9)	23 (2)	12 (9)	27 (4)	−10 (6)
C(8)	7557 (3)	7182 (6)	4760 (2)	58 (4)	73 (8)	26 (2)	−23 (9)	17 (5)	−9 (6)
O(8)	8750 (2)	6912 (5)	5621 (2)	49 (3)	153 (6)	21 (2)	6 (7)	−6 (3)	2 (5)
C(8A)	7846 (3)	7016 (6)	3404 (2)	44 (4)	60 (7)	29 (2)	−33 (8)	16 (5)	−18 (6)
C(9)	7858 (3)	5087 (6)	1118 (2)	50 (4)	95 (8)	25 (2)	−11 (9)	24 (5)	3 (7)
O(9)	6512 (2)	3951 (5)	1147 (2)	86 (3)	114 (6)	25 (2)	−90 (7)	24 (4)	−4 (5)
C(10)	9269 (4)	4798 (6)	2253 (3)	57 (4)	65 (8)	31 (2)	22 (9)	13 (5)	10 (6)

	X	Y	Z	β		X	Y	Z	β
H(1)	1035 (3)	633 (5)	389 (3)	1.5 (0.6)	H(6B)	293 (4)	827 (6)	482 (4)	4.4 (0.9)
H(3)	939 (3)	773 (5)	42 (2)	1.1 (0.6)	H(7A)	657 (4)	744 (5)	814 (3)	3.0 (0.8)
H(3A)	960 (4)	1096 (6)	112 (3)	2.7 (0.7)	H(7B)	774 (4)	723 (5)	713 (3)	2.4 (0.7)
H(3B)	778 (4)	1046 (5)	14 (3)	2.5 (0.7)	H(9)	822 (3)	484 (4)	25 (2)	0.6 (0.6)
H(3C)	801 (4)	1064 (5)	170 (3)	2.6 (0.7)	H(O9)	643 (4)	373 (5)	196 (3)	3.4 (0.8)
H(O4)	536 (4)	793 (6)	15 (3)	3.6 (0.8)	H(10A)	908 (3)	372 (4)	278 (2)	1.2 (0.6)
H(6A)	238 (4)	832 (5)	323 (3)	2.5 (0.7)	H(10B)	1041 (3)	458 (5)	196 (3)	1.9 (0.7)

Fig. 2. Structural formula of antibiotic U-58,431.

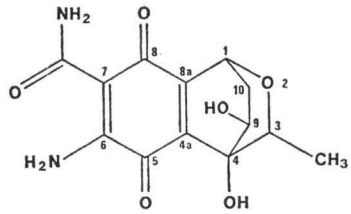
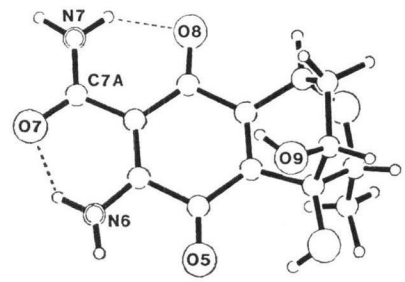


Fig. 3. Conformation and relative configuration of antibiotic U-58,431.



a=8.293(1)Å; b=7.492 (1) Å; c=10.543 (1)Å; β=99.58 (1)°; Dobs=1.48 gcm^{-3}; D_{calc}=1.51 cm^{-3}; μ (CuK)=9.3 cm^{-1}; 1139 reflections (999 reflections with intensities greater than three standard deviations).

Intensity data for all reflections with $2\theta < 138$°C were collected using the step-scan technique[2] at low temperature (−155°C) on a Syntex PI diffractometer controlled by an IBM 1800 computer using graphite monochromatized CuKα radiation (λ=1.5418 Å). Coordinates, including hydrogen coordinates, anisotropic thermal parameters of heavier atoms, and isotropic temperature factors of hydrogen atoms were refined by multiple matrix crystallographic least squares minimizing the function $\Sigma\omega(F_0^2 - F_c^{*2})^2$ where weights ω were taken as the reciprocals of the variances $\sigma^2(F_0^2)$ and where F_c^* was as defined by LARSON[4]. The final agreement index R[R=$\Sigma||F_0| - |F_c|/\Sigma|F_0|$] was 0.034, and the standard deviation of fit was 2.09. The final value of the secondary extinction parameter g was 13.6 (7)$\times 10^{-6}$. All calculations were carried out on an IBM 370 computer using the CRYM system of crystallographic programs written by DAVID J. DUCHAMP of The Upjohn Company.

Fig. 2 shows the structure of antibiotic U-58,431 and Fig. 3 shows the conformation and the relative configuration; the absolute configuration is not known. The intramolecular hydrogen bonds are also shown in Fig. 3; the intermolecular hydrogen bonds are: O9–(H)–O7, 2.734 (3) Å; N6–(H)–O2, 2.878 (3) Å; N7–(H)–O4, 2.928 (3) Å; O4–(H)–O9, 2.651 (3) Å, involving four other molecules. Final parameters and their standard deviations are given in Table 1; the numbering is as shown in Figs. 2 and 3.

VI. Biological Properties

Antibiotic U-58,431 inhibits a variety of Gram-positive and Gram-negative bacteria *in vitro* when assayed in a two-fold broth dilution test (Table 2). Particularly it is very active against *Streptococcus pneumoniae* (UC-41). The antibiotic is toxic to mice, having an MTD of 5

Table 2. *In vitro* antibacterial activity of antibiotic U-58,431.

Microorganism		Minimal inhibitory concentration* (mcg/ml)
Staphylococcus aureus	UC 76	125
Staphylococcus aureus	UC 570	125
Staphylococcus aureus	UC 746	125
Streptococcus hemolyticus	UC 152	125
Streptococcus faecalis	UC 694	250
Streptococcus pneumoniae	UC 41	1.0
Escherichia coli	UC 45	250
Proteus vulgaris	UC 93	250
Klebsiella pneumoniae	UC 58	125
Salmonella schottmuelleri	UC 126	62.5
Pseudomonas aeruginosa	UC 95	125

* Two-fold dilution end points in brain heart infusion broth. Read after 20 hours of incubation.

mg/kg/day when administered subcutaneously for four consecutive days and no protection of animals experimentally infected with *S. pneumoniae* was observed when they were treated at this level or below. Of fourteen fungi pathogenic to humans, only *Nocardia asteroides* and *Trychophyton rubrum* were inhibited by U-58,431 at the concentration of 1 mg/ml.

Added in proof

After submitting this paper, the editor of Journal of Antibiotics provided us with a galley of a manuscript describing a new antibiotic sarubicin A (REINHARDT, G., *et al.*, Isolation and characterization of sarubicin A, a new antibiotics. J. Antibiotics 33: 787~790, 1980). There is no doubt that antibiotic U-58,431 and sarubicin A are related. However, in view of slight differences in some physical properties, such as melting point and UV spectrum and the fact that the location of the amino and carboxamide groups in sarubicin A is not known, a direct comparison of the two substances would be desirable.

Acknowledgments

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